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Note

NMR analysis of galactoglucan from *Pseudomonas marginalis*: assignment of the ^{1}H and ^{13}C NMR spectra and location of succinate groups

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NMR analysis of galactoglucan from *Pseudomonas marginalis*: assignment of the ^{1}H and ^{13}C NMR spectra and location of succinate groups

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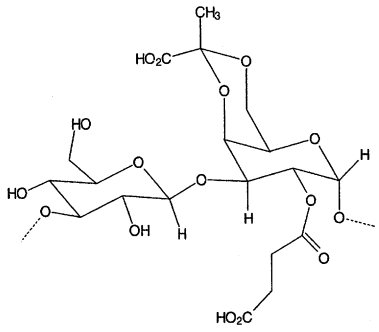
The exopolysaccharide (EPS) produced by *Pseudomonas marginalis* HT041B, an organism that causes spoilage of stored fruits and vegetables, has been already characterized as linear (1 → 3)-linked galactoglucan [1]. The repeating unit of this EPS (Scheme 1), also known by the trivial name marginalan, contains alternating 3-linked α-D-galactose and β-D-glucose residues and pyruvate and succinyl substituents in an approximate ratio 1:1:1:1. The pyruvate substituent was found to be acetalically linked to O-4 and O-6 atoms of the α-galactose unit, whereas the location of succinyl groups has not yet been precisely determined, although some predictions have been given [1]. In particular, it has been suggested that O-2 or O-4 of the glucose residues may be substituted [1].

The presence of succinyl groups is a feature that differentiates marginalan from other galactoglucans sharing the same structure of the repeating unit. In fact, bacteria isolated from water sediments (*Agrobacterium radiobacter*) and from root nodules of lucerne (*Rhizobium meliloti*) are able to produce galactoglucan, which instead of succinyl groups is substituted with acetyl ones in a variable amount [2–4]. In the case of



Scheme 1. Repeating unit proposed for EPS of *P. marginalis* [1], showing the refined location of the succinate group.

galactoglucan from *R. meliloti* Rm 1021, acetate was found to be linked at O-6 of the β-glucose unit [3].

Galactoglucans from different sources have been the subjects of several solution and solid-state investigations [5–7], but NMR spectroscopy was not extensively used for structural studies. To our knowledge, only one detailed ^{1}H NMR analysis was performed on chemically modified (substituent-free) galactoglucan from *R. meliloti* strain Rm 7011 exoA (pMuc) [8], but the proposed assignment was not rediscussed up to now. In the present work, in order to identify the location of succinyl groups and to provide the full ^{1}H and ^{13}C NMR assignment, we report the comparative NMR analysis of the native, and physically and chemically modified, samples of marginalan.

1. Results and discussion

Monodimensional experiments were initially carried out on the native marginalan (sample M). Broad linewidths of the signals, observed in both ^{1}H and ^{13}C NMR spectra, were related to the high molecular weight of the sample [9].

In the spectral region typical of non-carbohydrate substituents, the signals due to pyruvate and succinate groups were well resolved. In particular, in the ^{1}H and ^{13}C NMR spectra, the pyruvate CH_3 signal was present at δ 1.48 and 26.1, respectively. These values indicated, the *R* absolute configuration of the pyruvic acid acetal, as expected [1,10].

As previously reported for succinylated microbial exopolysaccharides [11], the succinyl substituent was found to be labile upon the thermal treatment. In particular, prolonged heating of the sample led to hydrolysis of succinate groups and accordingly in ^{1}H NMR spectra a sharp singlet appeared at δ 2.46 due to free succinic acid. In order to achieve a compromise between the resolution enhancement and temperature-induced removal of succinyl groups, two-dimensional (2D) NMR spectra were recorded at 323 K. A pH dependence of the spectral features of the succinate signal in the ^{1}H and ^{13}C NMR spectra of succinoglycans has already been described [12]. In the spectra of M, sonicated M and PF-M samples, a reversible change of a broad succinate singlet at pH

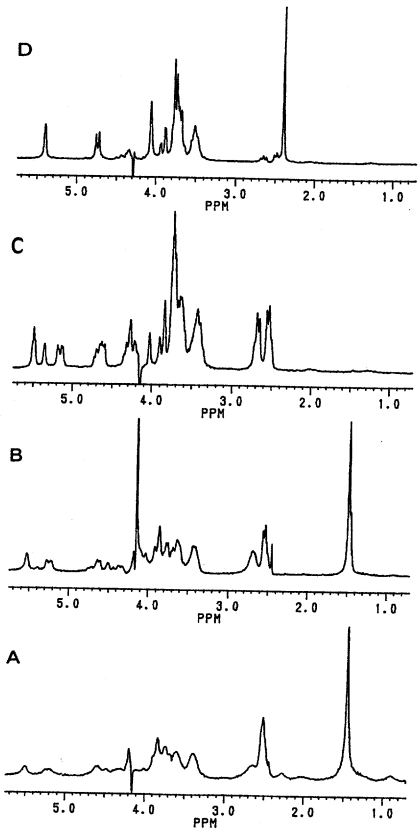


Fig. 1. ^{1}H NMR spectra (200 MHz) of: A, M sample at 353 K; B, sonicated M sample at 353 K; C, PF-M sample at 353 K; D, PSF-M sample at 343 K.

~ 5 (δ 2.64 and 30.5) to two broad signals at pH 6.5–7.0 ($\delta \sim 2.66$, 2.53 and δ 31.9, 31.0, respectively) was also detected. However, in contrast to succinoglycans at neutral pH [12], the intensity ratio of two succinate signals in the ^{1}H NMR spectrum of M was 1:2 (Fig. 1A) and it was found to be molecular-weight and temperature dependent. In a first approximation, this peculiar behavior might be explained by different interactions of the main chain-linked succinate of marginalan in comparison with relatively free side chain-linked succinyl substituent in succinoglycan. The behavior of the succinate substituents will be a subject of further investigations.

From the ^{1}H NMR spectrum of the native M sample, the ratio between pyruvate and succinate was estimated to be 1:0.7, indicating a partial succinylation of the polymer.

In the anomeric region of the ^{1}H NMR spectrum of the native polymer the α-Gal H-1 signal could be easily recognized as a broad singlet at δ 5.52. The region around δ 4.60 at which β-Glc H-1 was expected, was less resolved and showed a pattern indicating overlapping of several signals (Fig. 1A). Moreover, an unidentified signal at δ 5.24 was also present. On the basis of our experience with succinylated microbial EPS this signal

was expected to be downfield shifted because of the succinylation [12]. However, the 2D COSY experiment, used to determine through-bond connectivities, failed to clarify the origin of this signal because of the poor resolution. For this reason sample M was partially depolymerized by means of sonication.

For the sonicated M sample, no change in the ratio between pyruvate and succinate was detected in comparison with the native polymer and the enhanced resolution was achieved especially in the ^{1}H NMR spectra (Fig. 1B and Fig. 2A). In addition to the signals observed in the anomeric region of the M sample, the ^{1}H NMR spectrum of the sonicated polymer showed the β-Glc H-1 signal at δ 4.63 with $J_{1,2} \sim 7$ Hz in the crowded region of δ 4.80–4.30 as reported in Fig. 1B. Moreover, the cross-peak signal of α-Gal H-1 in the 2D COSY spectrum (Fig. 2A) revealed a connection with the signal at δ 5.24, previously unidentified. This finding strongly supports the location of the succinate groups at position 2 on the galactose residue. The presence of other signals due to α-Gal H-1 (δ 5.40 $J_{1,2} \sim 1$ Hz) and β-Glc H-1 (δ 4.74, $J_{1,2} \sim 7$ Hz) with small intensities could be also detected. Even though these signals were expected to belong to non-succinylated polymer fragments, the complex spectral pattern around $\delta \sim 4.50$ prevented the unequivocal exclusion of the presence of further downfield shifted signals due to additional sites of succinylation.

Further studies were carried out by investigating a depyruvated sample (PF-M). The depyruvation of the native polymer permitted the determination of the effect of pyruvate groups upon the chemical shifts of α-Gal residues to which they are linked. In parallel, the chemical treatment leading to depyruvation substantially decreased the molecular weight of the polymer, as suggested mainly by an enhanced resolution in 1D and 2D ^{1}H NMR spectra (Fig. 1C and Fig. 2B).

The occurrence of two different α-Gal residues in the polymer chain was comfirmed by the presence of two anomeric signals at δ 5.51 and 5.40, in full agreement with the sonicated M sample. The ratio (2:1) indicated that one of these α-Gal residues predominates.

In order to better understand this feature, the removal of the succinyl groups from the PF-M sample was performed directly in the NMR sample tube while monitoring the reaction. The spectra clearly showed that in the course of the hydrolysis the intensity of the predominant α-Gal H-1 signal at δ 5.51 regularly decreased, while the minor signal at δ 5.40 regularly increased.

These results gave further evidence that the succinate groups are located on the galactose residue and that the anomeric signal at δ 5.51 refers to the succinylated galactose units while the other one at δ 5.40 is related to non-substituted galactose residues. In addition, the proportion of these anomeric signals indicated the partial succinylation of the polymer, in good agreement with the above-mentioned ratio of pyruvate and succinate signals found for the native and sonicated M samples.

The ''triplet'' structure of the α-Gal H-1 signal at δ 5.51 in the spectrum of PF-M sample observed at lower field (200 MHz) (Fig. 1C) was resolved to two close doublets at higher field (300 MHz) with δ 5.53 and 5.50. Their intensity changing in the series of spectra recorded to monitor the hydrolysis of succinyl substituents suggested their interpretation. The α-Gal H-1 signal at δ 5.50 was decreasing its intensity and was ascribed to the α-Gal residue of succinylated repeating units neighboring succinylated

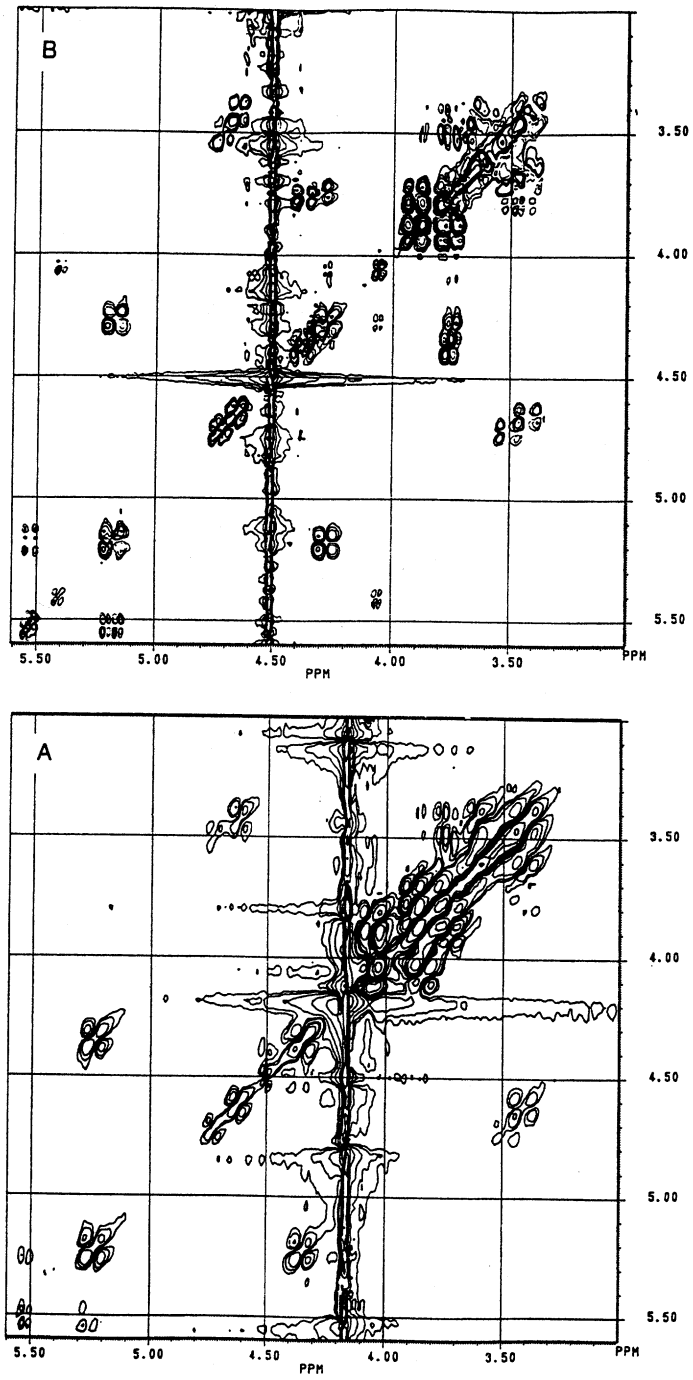


Fig. 2. 2D phase-sensitive COSY spectra of: A, sonicated M sample (353 K); B, PF-M sample (323 K).

Table 1
^{1}H NMR data obtained from all types of experiments for polysaccharides investigated

EPS	Residue	Chemical shifts δ/ppm								
		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	Pyr	Succ.
PSF-M	α-Gal	5.40	4.06	4.07	4.28	4.30	3.74	3.74		
	β-Glc	4.73	3.51	3.70	3.73	3.51	3.92	3.76		
PF-M	α-Gal	5.51	5.17	4.27	[a]	[a]	3.75	3.75		2.70, 2.54
	β-Glc	4.66	3.43	3.65	3.65	3.45	3.89	3.74		
M	α-Gal	5.53	5.24	4.36	4.49	4.24	4.87	3.71	1.48	2.66, 2.53
	β-Glc	4.60	3.40	3.63	3.63	3.42	3.95	3.76		

[a] Overlapped at δ 4.40–4.30.

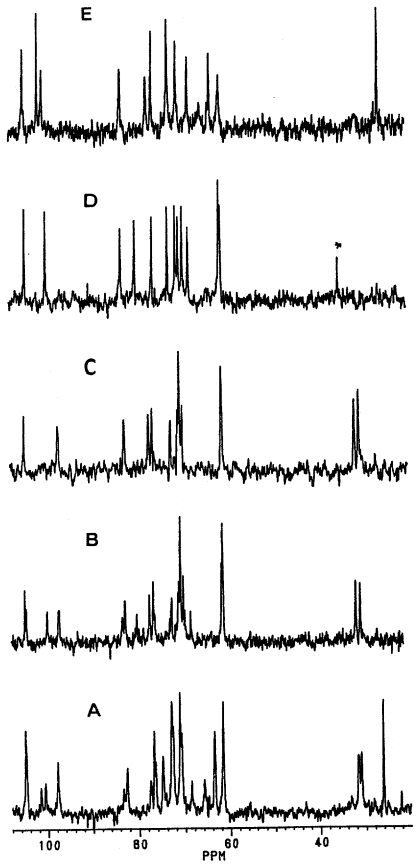


Fig. 3. ^{13}C NMR spectra (300 MHz) of: A, M sample at 353 K; B, PF-M sample at 323 K; C, difference spectrum of the PF-M sample at 323 K, for details see the text; D, PSF-M sample at 323 K (*, free succinic acid); E, SF-M sample at 353 K. The region of carbonyl atoms not shown.

repeating units. On the other hand, α-Gal H-1 signal at δ 5.53 was attributed to the α-Gal residue of succinylated repeating units neighboring the non-succinylated repeating units.

The spectral pattern of β-Glc H-1 was also affected by the presence of two types of repeating units, resulting in the signals overlapping. In fact, in the 2D COSY spectra of sonicated M and PF-M (Fig. 2A and 2B) two cross peaks H-1/H-2 with different intensities for β-Glc were detected. The whole set of ^{1}H NMR data (Table 1) indicated that the chemical-shift changes of the protons belonging to glucose residues induced by the chemical treatments were negligible in comparison with those of the galactose residues, thus strongly suggesting no substitution on glucose residues.

The removal of the succinate groups from the PF-M sample led to an unsubstituted galactoglucan (PSF-M sample) sharing the same repeating unit of the depyruvated (1 → 3)-linked galactoglucan from *R. meliloti* previously investigated by Levery et al. [8] by means of ^{1}H NMR spectroscopy. However, the data reported by Levery et al. were not in accordance with our PSF-M ^{1}H NMR data. This prompted us to reinvestigate the assignment of the SF-M sample. Surprisingly, the obtained data of SF-M sample fitted (were in agreement) with the data reported by Levery et al. It might be

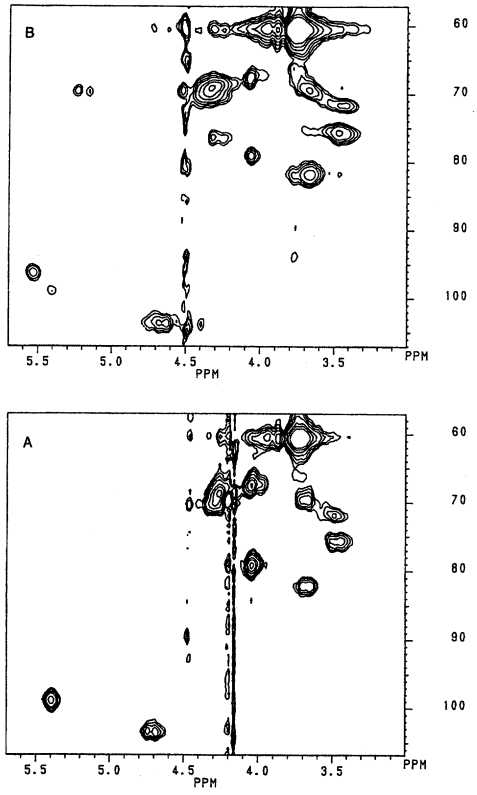


Fig. 4. HMQC spectra (323 K) of: A, PSF-M sample; B, PF-M sample.

Table 2
^{13}C NMR data of polysaccharides investigated

EPS	Residue	Chemical shifts δ (ppm)										
		C-1	C-2	C-3	C-4	C-5	C-6	Pyr C=O	Pyr CH_3	Pyr C	Succ. C=O	Succ. CH_3
PSF-M	α-Gal	99.8	68.5	80.1	69.8	71.3	61.6					
	β-Glc	104.5	73.0	83.1	70.7	76.4	61.3					
SF-M	α-Gal	100.6	68.5	77.6	73.0	63.7	65.8	176.4	26.1	101.6		
	β-Glc	104.9	73.0	83.2	71.1	76.4	61.6					
	$\Delta\delta$	+0.8	0.0	−2.9	+2.2	−7.6	+4.2					
PF-M [a]	α-Gal	97.3	70.7	77.4	70.0	71.0	61.4				×	31.5, 30.6
	β-Glc	104.8	72.6	82.6	70.7	76.6	61.3					
	$\Delta\delta$	−2.5	+2.2	−3.1	+0.2	−0.3	−0.2					
M [b]	α-Gal	97.9	70.5	75.3	72.4	63.4	65.7	176.5	26.1	101.7	180.9, 175.6	31.9, 31.0
	β-Glc	105.3	72.9	82.4	71.1	76.6	61.5					
	$\Delta\delta$	−1.8	+2.0	−5.2	+2.6	−7.9	+4.1					

[a] Data obtained after subtraction of the spectra (for details see the text).
[b] Sonicated marginalan sample.
× Not assigned; $\Delta\delta$ difference in the chemical shifts of given sample and PSF-M sample.

possible that Levery et al. performed a partial depyruvation of their product but not a total removal of pyruvate groups as they claimed and so the reported ^{1}H NMR data are valid for 4,6-*O*-pyruvated (1 → 3)-linked galactoglucan. Unfortunately the upfield region of the spectrum reported by Levery et al. is not shown [8].

In full agreement with ^{1}H NMR data, the ^{13}C NMR spectra of the M, sonicated M, and PF-M samples, showed two different α-Gal C-1 signals due to succinylated and non-succinylated units (Fig. 3). Furthermore, in agreement with the location of succinate groups on O-2 of the galactose residues, only one C-1 signal of β-Glc was detected. In order to refine the preliminary assignment of the ^{13}C NMR spectrum of the native marginalan previously reported [1], as well as to assign the carbon spectra of all polysaccharides used in the present study, heterocorrelated HMQC spectra were measured (Fig. 4). The signals in the ^{13}C NMR spectrum of the sonicated M sample were assigned from two HMQC spectra. The second short experiment (353 K) was necessary to shift a residual water signal and to reveal the cross peaks of interest.

The complete set of ^{1}H and ^{13}C NMR data (Tables 1 and 2) supporting this finding also enabled an evaluation of the effect of the succinate as well as pyruvate substituents on the chemical shifts. In addition to the α-effect of succinyl groups on Gal H-2 ($\Delta\delta$ +1.1) observed in the ^{1}H NMR spectra of M and PF-M samples, chemical shifts changes in the ^{13}C NMR spectra also provide evidence about the unusual location of this bulky succinate substituent. For the PF-M sample α-effect on Gal C-2 ($\Delta\delta$ +2.2) as well as β-effects of succinyl groups on the chemical shifts of Gal C-1 and C-3 ($\Delta\delta$ −2.5 and −3.1, respectively) were found. These values are consistent with the α- and β-effects of the acetyl group [13,14]. For sample M, in addition to the β-effect of the succinyl group on the chemical shift of Gal C-3 the β-effect of 4,6-*O*-linked pyruvate is also present (Table 2).

The distribution of the non-succinylated α-Gal residues influences significantly the spectral pattern and this is particularly remarkable in the case of the random distribution. In order to obtain preliminary information, a mathematical subtraction of the spectrum of PSF-M sample (Fig. 3D) from that of the PF-M sample (Fig. 3B) was performed. This strategy permitted the removal of the signals of the non-succinylated (1 $\rightarrow$ 3)-linked galactoglucan segment from the spectrum. The obtained difference spectrum (Fig. 3C) contained only the signals of the succinylated (1 $\rightarrow$ 3)-linked galactoglucan segments, with the signal intensities in full agreement with the number of carbon atoms per repeating unit. This result suggests in the first approximation a regular distribution of non-succinylated α-Gal residues, e.g., an alternation of large succinylated and short non-succinylated segments in the polymer chain.

2. Conclusions

Two unexpected results were found in the present investigation. Taking into account that the galactose residues are substituted at O-4 and O-6 positions with pyruvate groups, the first of them was the location of succinate found on O-2 of the galactose residues. A discrepancy in the degree of succinylation between this batch of marginalan and the one previously characterized [1] was the second unexpected result. These aspects, together with the biological implications of the particular location of succinate groups on marginalan, are matters for further investigation.

3. Experimental

Materials.—Marginalan was isolated from a culture of *P. marginalis* HT041B and purified as previouly described [1]. The polymer was solubilized in Milli-Q water (polymer concentration 0.1% w/v) at 25 °C with stirring. The resulting solution, showing the presence of aggregates, was centrifuged (10,000 rpm, 30 min, 20 °C), the supernatant recovered and NaCl was added in order to achieve 1 M salt concentration. After neutralization and exhaustive dialysis against Milli-Q water, the sample was recovered by freeze-drying to give white fluffy material (designated hereafter as sample M).

In order to decrease the molecular weight of sample M without chemical modification, an aqueous solution (Cp = 2.0 g/L) of the native sample was sonicated for 4.5 h at maximum energy by using a MSE Soniprep 150 ultrasonic desintegrator. A succinate-free marginalan sample (designated hereafter as sample SF-M) was prepared as previously described [12].

In order to remove the pyruvate groups and to decrease the molecular weight of the native polymer, dilute HCl was added to an aqueous solution (Cp = 1.6 g/L) to give a final pH of 2.5 and the solution was heated at 100 °C with stirring under N_2. Three aliquots of the solution were obtained after 2, 6, and 10 h of heating in order to monitor the efficiency of the depyruvation reaction. Each aliquot was then cooled to room temperature, neutralized, and dialyzed exhaustively against Milli-Q water. The

pyruvate-free, partially depolymerized marginalan (designated hereafter as PF-M) was recovered by freeze-drying.

Desuccinylated-depyruvated marginalan (designated hereafter as PSF-M) was prepared following two different procedures: (1) directly in a NMR sample tube by addition of NaOD to the PF-M sample solution and additional heating; (2) by adding dilute NaOH to an aq PF-M sample solution (Cp = 1.5 g/L) to give a final pH of 12.5 which was maintained for 2 h at room temperature with stirring under N_2. The sample was recovered, after neutralization and exhaustive dialysis against Milli-Q water, by freeze-drying.

Methods.—Samples of the native and sonicated marginalan for ^{1}H NMR spectroscopy (200 MHz) were treated with D_2O, lyophilized, redisolved in D_2O in a 5-mm sample tube and sealed. ^{13}C NMR spectra (300 MHz) were measured in a 10-mm sample tube. All 2D NMR experiments were performed at 323 K, if not stated otherwise, on Bruker AC 200 (^{1}H, 200.13 MHz and ^{13}C, 50.33 MHz) and AM 300 WB (^{1}H, 300.13 MHz and ^{13}C, 75.47 MHz) spectrometers, equipped with a process controller and an Aspect 3000 computer. ^{1}H and ^{13}C chemical shifts are referenced to internal sodium 4,4-dimethyl-4-sila pentanoate-2,2,3,3-d_4 (δ 0.00 ppm) and external acetone (δ 31.07 ppm), respectively. 2D phase-sensitive COSY spectra were obtained using the method of time-proportional phase incrementation (TPPI) [15] with double quantum filtering [16,17]. For all 2D ^{1}H NMR experiments, typical parameters were 1400 Hz spectral width, 1 K $\times$ 256 time-domain data matrix zero-filled to 1 K $\times$ 512, 80–160 scans for each FID with relaxation time of 1.5 s. A Lorentz–Gauss multiplication of the FID signals was used prior to Fourier transformation (FT). Quantitative 1D ^{13}C NMR spectra were acquired with inverse-gated decoupling for NOE suppresion using a relaxation delay of 2 s and an acquisition time of 0.5 s. ^{1}H-^{13}C correlation spectra were obtained in inverse mode (^{1}H detection) using the HMQC pulse sequence [18]. Spectral widths were of 850 and 3500 Hz in F2 and F1, respectively, were used with the time domain data matrix 1 K $\times$ 128 zero-filled to 1 K $\times$ 256, 1600–4000 scans for each FID were accumulated with a relaxation delay 0.5–1 s. A Lorentz–Gauss function was applied in both dimensions prior to FT. 1D TOCSY experiments were performed by selective spin echo excitation [19] using DANTE pulse trains [20] followed by a pulsed MLEV-17 sequence for spin lock (mixing times 21–95 ms), using the transmitter in the low power mode.

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